Frontiers in Environmental Microbiology
2018; 4(5): 115-123
http://www.sciencepublishinggroup.com/j/fem
doi: 10.11648/j.fem.20180405.11
ISSN: 2469-7869 (Print); ISSN: 2469-8067 (Online)

Biodegradative Potentials of Phytase-producing Bacterial Isolates Recovered from Spent Engine Oils Polluted-Soils

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To cite this article:
Osunla Charles Ayodeji. Biodegradative Potentials of Phytase-producing Bacterial Isolates Recovered from Spent Engine Oils Polluted-Soils. *Frontiers in Environmental Microbiology*. Vol. 4, No. 5, 2018, pp. 115-123. doi: 10.11648/j.fem.20180405.11

Received: December 9, 2018; **Accepted**: January 2, 2019; **Published**: January 29, 2019

Abstract: Microbial remediation of environmental contaminants such as spilled and used petroleum products is an increasing auspicious technique, owing to its associated low-cost and eco-friendly outcomes compared to other methods. For this purpose, recovered bacteria isolates from contaminated soils in automobile workshops were screened for phytase activity and hydrocarbon biodegradative ability. Presumptive bacterium with inherently high phytase activity and biodegradative potential was further characterized using 16S rRNA gene sequence analysis. Soil total petroleum hydrocarbon (TPH) was determined using gas chromatographic technique (GC-FID). The identities of the isolates recovered from the samples include; *Bacillus subtilis, B. licheniformis, Pseudomonas aeruginosa, Escherichia coli, Corynebacterium variabilis, Micrococcus luteus and Proteus vulgaris*. Of all the isolates, *P. aeruginosa* had the highest phytase activity after 48 h of incubation whereas, *P. vulgaris* recorded the least phytase activity. *E. coli* and *B. subtilis* showed active phytase activity at pH 5.0 and 40°C. While *P. aeruginosa* exhibited proficient degrading ability on crude oil and spent engine oil at all days of incubation, *E. coli* and *C. variabilis* showed the most inaptitude. The 16S rRNA gene analysis shows that the isolate obtained from the automobile workshop is of the genus *P. aeruginosa* with reference to ATCC 27853. The TPH of the contaminated soils ranged from 545,168 to 856,328 Mg/kg. This study reveals the degradative potential of *P. aeruginosa* as suitable candidate in bioremediation of crude oil contaminated sites.

Keywords: Bacteria, Biodegradation, Bonny Light Crude Oil, Phytase

1. Introduction

Crude oil contamination of soil and water have been a major threat to human and ecosystem through the transfer of toxic organic materials including saturated and polycyclic aromatic hydrocarbons (PAHs) into the environment [1]. Presence of polycyclic aromatic hydrocarbons (PAHs) in soil and water constitute environmental problems because of their recalcitrant and toxic nature. Biodegradation involves the use of metabolic ability of microorganisms to transform or mineralize organic contaminants into less harmful substances, many of which are integrated into natural biogeochemical cycles [2, 3]. In recent years, bacteria have been considered as the most predominant hydrocarbon degrading microorganisms found in contaminated environment, which are free living and ubiquitous [4]. Microbial populations that are exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes in the contaminated soil or water [5]. The indigenous microflora are known to exhibit higher biodegradation rates than non-indigenous microorganism in hydrocarbon contaminated environment. Bioremediation of crude oil polluted soil relies on the crude oil degradation ability of the microbial consortium resident in the soil [6, 7]. Although petroleum-degrading microorganisms are widely distributed in both soil and water, but not in sufficient numbers at a given polluted site. In such cases, it may be helpful to inoculate the polluted area with highly efficient petroleum-degrading microbial strains in a process called bioaugmentation [8].

Phytase is an enzyme that catalyzes the hydrolysis of phytic acid. [9] reported the presence of phytase in microorganism, plants and animals. However, plants have been found to show only limited phytase activity to acquire phytate P form soil, on their own [10]. On the other hand, microorganisms are considered the best source of phytase production even on commercial scale. Bacterial phytases

have more alternative uses than other known sources because of its properties such as proteolysis resistance, thermal stability, resistance against metal ions, commercial substrate specificity and high catalytic activity [11]. Furthermore, phytase source from fungi increases the bioavailability of phosphorus contained in oil contaminated soil [12], and in the straw of raw feed [13]. There are many applications of phytase which includes corrosion inhibitor on metals, combating environmental phosphorus pollution and as additive in lubricating greases; food additive, and medical applications, including use in the prevention of dental caries, imaging agent for organ scintigraphy and X-ray contrast enhancing agent [14]. However, this study was designed to isolate and also optimize culture conditions for phytase-producing bacterial isolates with biodegradative potentials.

2. Materials and Methods

2.1. Collection of Sample

Oil contaminated and non-contaminated (control) soil samples were collected from three different mechanic workshops at three random spots using a soil auger in Akure, Ondo State Nigeria. Soil samples for microbiological analysis were collected in sterile screw-capped bottles. Analysis commenced immediately upon arrival in the laboratory. Unused samples were refrigerated at 4°C.

2.2. Isolation of Phytase Hydrolyzing Bacteria

Soil bacteria were isolated according to [15]. Briefly, 1.0g of the soil samples was serially diluted in 9ml of sterile saline solution, briskly shaken for about 30 mins and selected diluents were plated on Luria-Bertani (LB) plates (LB; 10 g/L D-glucose, 5 g/L yeast extract, 10 g/L tryptone and 15g Agar) and incubated at 37 ^{o}C for 24 h. The pure colonies obtained were repeatedly subcultured on LB plates supplemented with 4g/L Na-phytate 37 ^{o}C for 16 h to obtain pure isolates which were characterized to genus level based on colony morphology and pigmentation and biochemical tests. All the pure colonies recovered were examined for their ability to hydrolyze phytate on the phytase screening medium (PSM) agar (PSM; 10 g/L D-glucose, 4 g/L Na-phytate, 2 g/L $CaCl_2$, 5 g/L NH_4NO_3, 0.5 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$, 0.01 g/l $MnSO_4.H_2O$) according to [16]. Counter staining technique as described by [17], was used in vitro phytase activity assay. Moreover, considering the phytase hydrolyzing activity, appraised based on the clear halo zone (diameter) onto PSM media, seven efficient phytase-producing bacteria were selected for characterization.

2.3. Determination of Phytase Production

Extracellular phytase production was accessed in bacterial culture grown in PSM and determined as described by the method of [18]. The reaction mixture in a test tube contained 1 ml of the crude enzyme solution, 2 ml of of 5 mM Na-phytate (in 0.1 M citrate buffer, pH 5.5) incubated at 37°C for 65 min using a regulated Gallenhamp water bath and the reaction was stopped with freshly prepared 2 ml of colour stop mix to precipitate the enzymes. Blank sample contained only 1 ml of sodium acetate buffer, 2 ml of substrate and 2 ml of colour stop mix. Colour stop mix consist mixture of ammonium molybdate stock solution, ammonium vanadate stock solution, nitric acid ($HN0_3$) and distilled H_20. The change in the colour developed from Phytase activity (phosphorous released) was determined at absorbance at 415 nm in a Spectrophotometer. One enzyme unit (IU) was defined as 1 µmol phosphate liberated per minute.

2.4. Effect of pH on the Activity of Phytase

Two bacteria, *Bacillus subtilis* and *Escherichia coli* were selected for this assay. The optimal pH was determined by measuring the activity pH range 4.0-8.0, using 0.1 M acetate (pH 4.0-5.0), 0.1 M phosphate (pH 6.0-7.0) and 0.1 M Tris (Ph 8.0) buffers. Maximum activity was taken as the optimum pH for phytase for activity expressed in comparison with maximum activity [19].

2.5. Effect of Temperature on Activity of Phytase

The temperature profiles of the selected phytase producing microorganisms were carried out by incubating the assay mixture at different temperature ranges from 30-60°C. At 65 min incubation period, 2 ml colour stop mix was added and the enzyme activity was measured according to the standard assay method at 5°C interval for each of the different temperature.

2.6. Determination of Rates of Utilization of Crude Oil and Used Engine Oil by Bacterial Isolates

Minimal salts medium (MSM) of [20] containing; 1% refined petroleum product (crude oil and used engine oil) as the only source of carbon, 0.27 g K_2HPO_4, 0.6 g NH_4Cl, 0.03 g $MgSO_4.7H_2O$, 0.015 g NaCl, 0.0015 g $NaSO_4.7H_2O$ was used. Crude oil and used engine oil were tested directly for the ability of fungal and bacterial isolates to degrade them using the method earlier described by Okpokwasili and Okorie (1988), as their sole sources of carbon and energy by the determination of growth turbidity. This was carried out by dispensing 100 ml of MSM into conical flask (Zajic and Supplison, 1972). Following sterilization by autoclaving and cooling, 0.1 ml of the isolates from 10-4 to 10-6 dilutions were seeded in 1,000 ml of minimal salt medium, pH 7.4, medium, followed by 0.1 ml filter-sterilized (0.45 µm pore size filter, Millipore) crude oil and used engine oil. The cultures were then incubated at room temperature for seven days. For each isolate, a control was set up in which no organism was seeded. At the end of the incubation, the optical density (OD) of each culture was measured at 650 nm [21] using spectronic 20 Genseys spectrophotometer. In this case, the OD was an index of growth reflecting the potential for the biodegradation of the petroleum products by the respective fungal species.

2.7. Characterization of Crude Oil and Used Engine Oil

The crude oil sample (Bonny light), used engine,

contaminated and non-contaminated soil sample were subjected to total hydrocarbon analysis using gas chromatographic (GC) method to determine total petroleum hydrocarbon (TPH) content of the crude oil and engine oil sample according to the method of [22].

2.8. Molecular Charactarization of Pseudomonas aeruginosa

Conserved regions within the 16S rRNA gene were used as target sequences to confirm the identities of the selected presumptive *Pseudomonas aeruginosa.* DNA extraction was carried out using the boiling method as described by [23]. Polymerase chain reaction (PCR) amplification of the 16 rRNA was carried using the primer pair P Ps-F (5'-GGT CT GAGA GGA TGA TCA GT-3') and Ps-R (5'-TTA GCT CC ACCT CGC GGC-3'). The DNA from *Pseudomonas aeruginosa* ATCC 27853 was used as positive control. Thermal cycling conditions used is as follow: initial denaturation of 95°C for 5 minutes followed by 30 amplification cycles of 30 seconds at 95°C; 1 minute at 52°C and 1 minute at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was resolved on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. A 100bp DNA ladder was used as DNA molecular weight standard.

2.9. Statistical Analysis of Data Obtained

Data obtained were subjected to a single factor analysis of variance (ANOVA) while the significant means were separated with the Duncan's new multiple range test (DNMRT) at 5% confidence level (P = 0.05) using SPSS (16).

3. Result

3.1. Total Plate Count of Bacteria in Uncontaminated and Contaminated Soils

The total plate counts of bacteria were higher in uncontaminated soils than in contaminated soils. It ranged from 2.0×10^5 to 3.0×10^5cfu/g in contaminated soils and 3.0×10^5 to 5.0×10^5 cfu/g in uncontaminated soils. Seven bacterial isolates; *M. luteus, B. licheniformis, E. coli, P. aeruginosa, B. subtilis, P. vulgaris,* and *C. variabilis* were obtained from both contaminated and non-contaminated soil samples.

3.2. Growth Profile of Isolated Bacteria

In Figure 1, there was an exponential phase at 15 to 50 h. of incubation, *Bacillus licheniformis* had 15 to 55 hrs of incubation, *B. subtilis* had 22 to 45h. of incubation and *P. aeruginosa* had 20 to 50 h. of incubation.

3.3. Production of Phytase

Result of phytase activity from isolated bacteria is presented in Figure 2. From this study, it was observed that of all the bacterial isolates, *P. aeruginosa* had the highest phytase activity at 50 h of incubation while *P. vulgaricus* produced the least phytase activity at all the hour of incubation. Phytase activity of *B. subtilis* and *E. coli* were found to be most active at pH 5.0 (Figure 3). Also at 40°C, phytase activity of *B. subtilis* and *E. coli* were found to be at optimum (Figure 4).

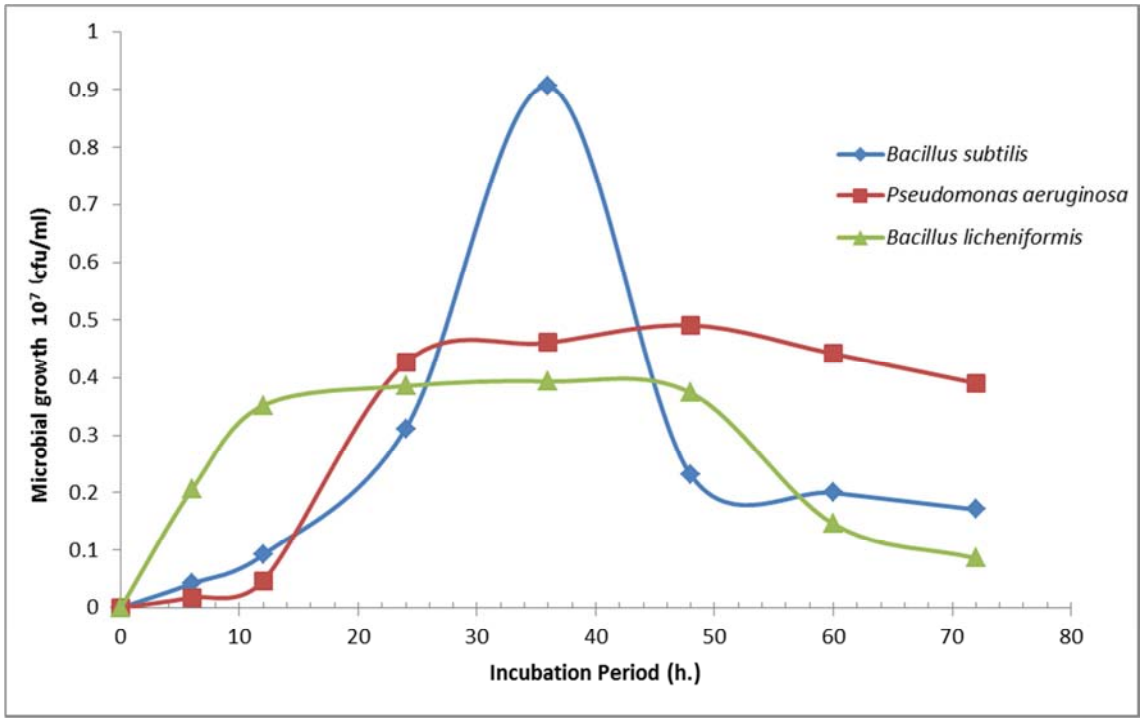


Figure 1. *Growth profile of selected bacterial isolates in optimized nutrient medium.*

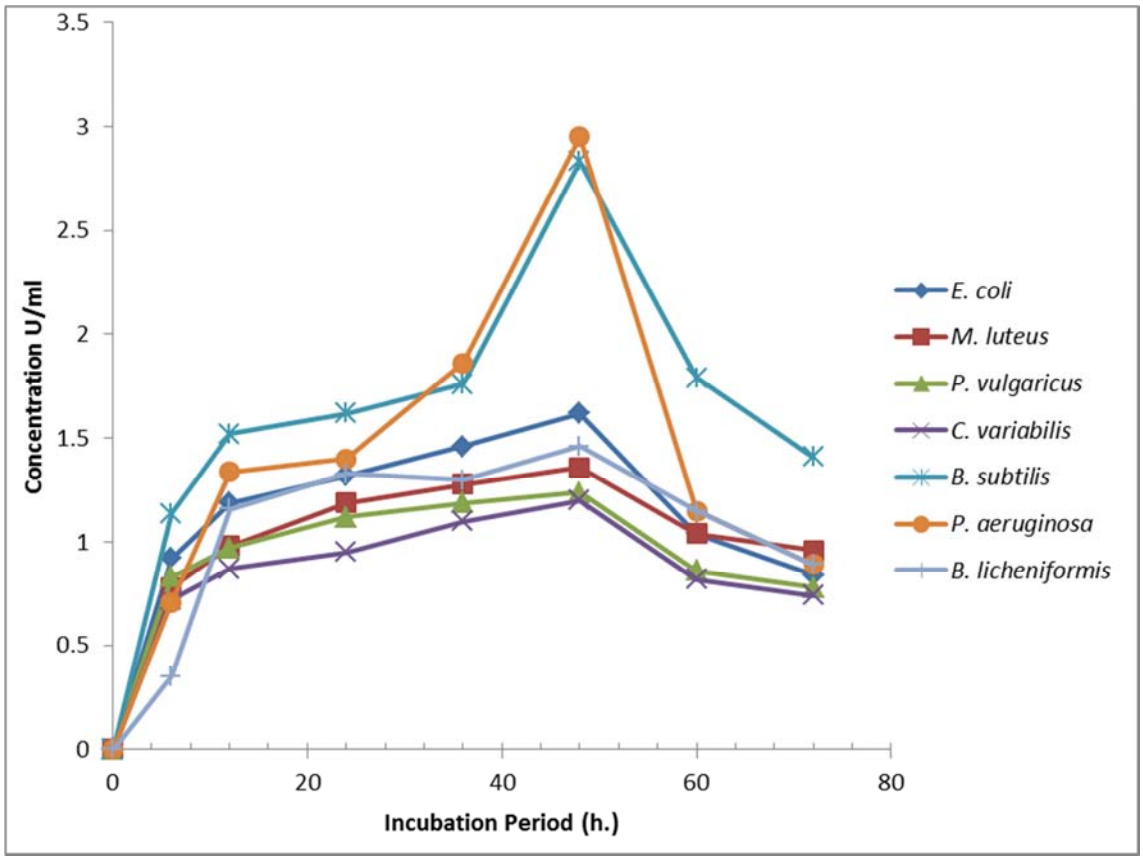


Figure 2. *Phytase activity of soil bacterial isolates from oil contaminated mechanic workshop.*

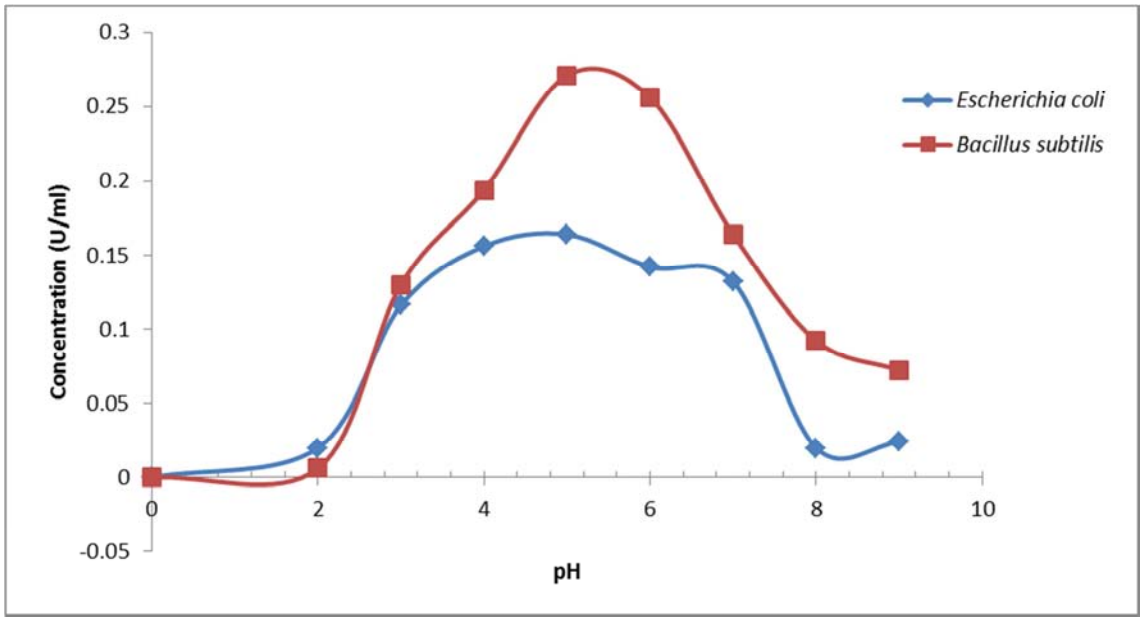


Figure 3. *Effect of pH on the activity of phytase from E. coli and B. subtilis.*

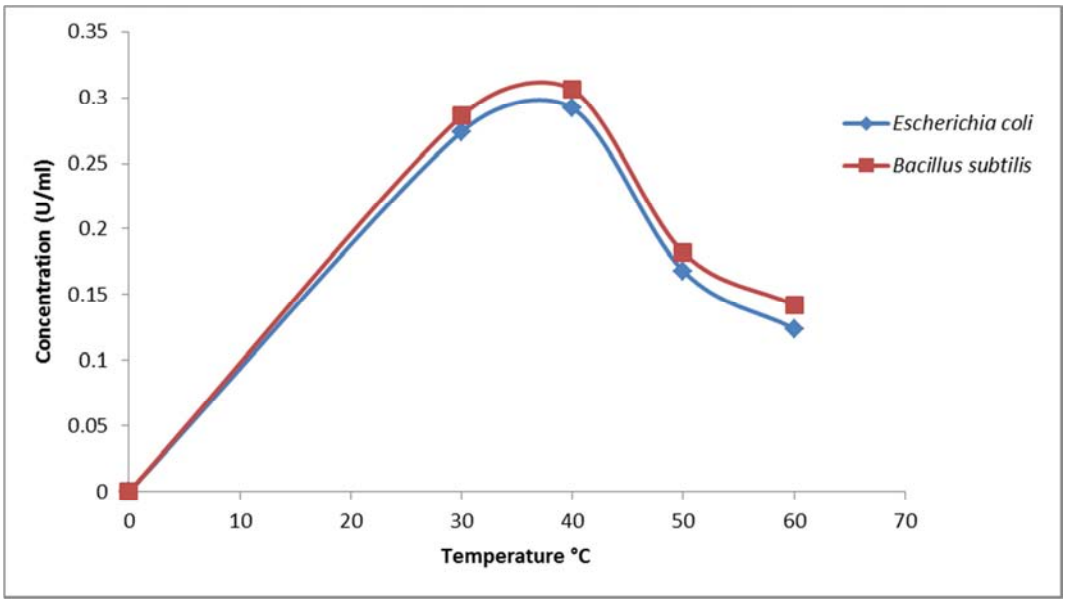


Figure 4. *Effect of temperature on the activity of phytase from E. coli and B. subtilis.*

3.4. Biodegradation of Bonny Light Crude Oil and Used Engine Oil by Bacterial Isolates

The degradative abilities of bacterial isolates on crude and used engine oil are as shown in Figures 5, 6, 7 and 8. Degradative abilities of crude oil were observed to be in the following order: Degradative abilities of crude oil were observed to be in the following order: *P. aeruginosa* > *B. subtilis* > *B. licheniformis* > *P. vulgaricus* > *E. coli* > *M. luteus* > *C. variabilis*. On the other hand, the abilities of bacteria to degrade used engine oil were observed in the following order: *B. subtilis* > *P. vulgaricus* > *P. aeruginosa* > *B. licheniformis* > *M. luteus* > *C. variabilis* > *E. coli*. *P. aeruginosa* was observed to be the best bacterium for rapid degradation of crude oil and *B. subtilis* for used engine oil while *C. variabilis* and *E. coli* were observed to be the least degrader of both crude oil and used engine oil respectively.

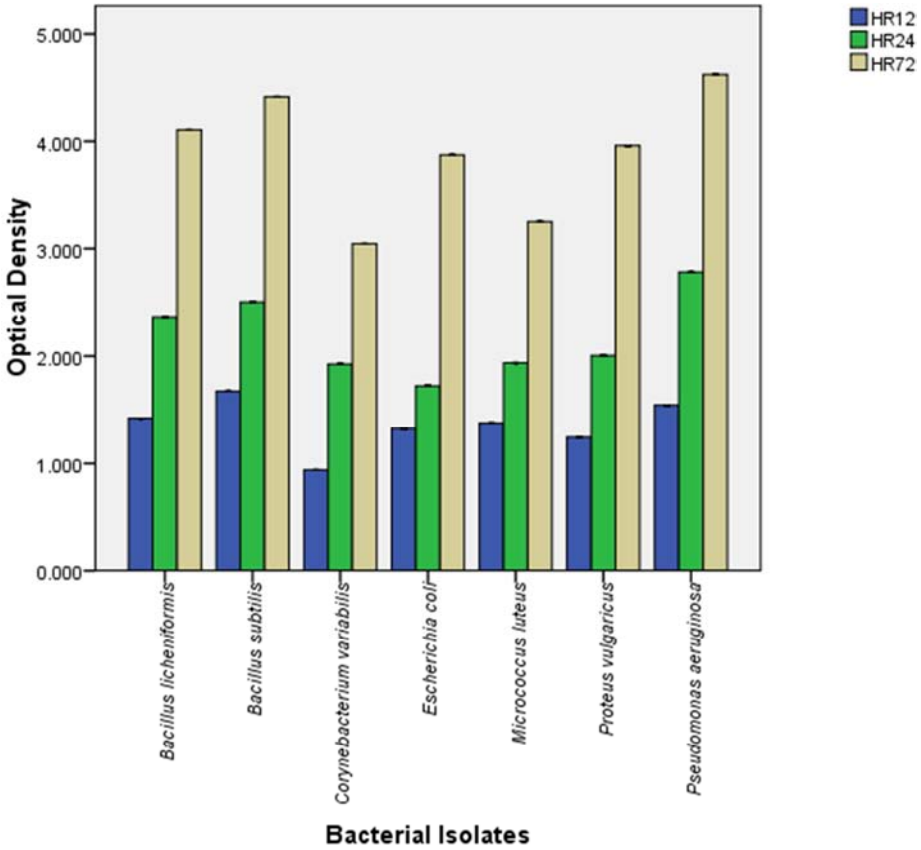


Figure 5. *Optical density of bacterial isolates from contaminated soil at different hours of biodegradation of bonny light crude oil.*

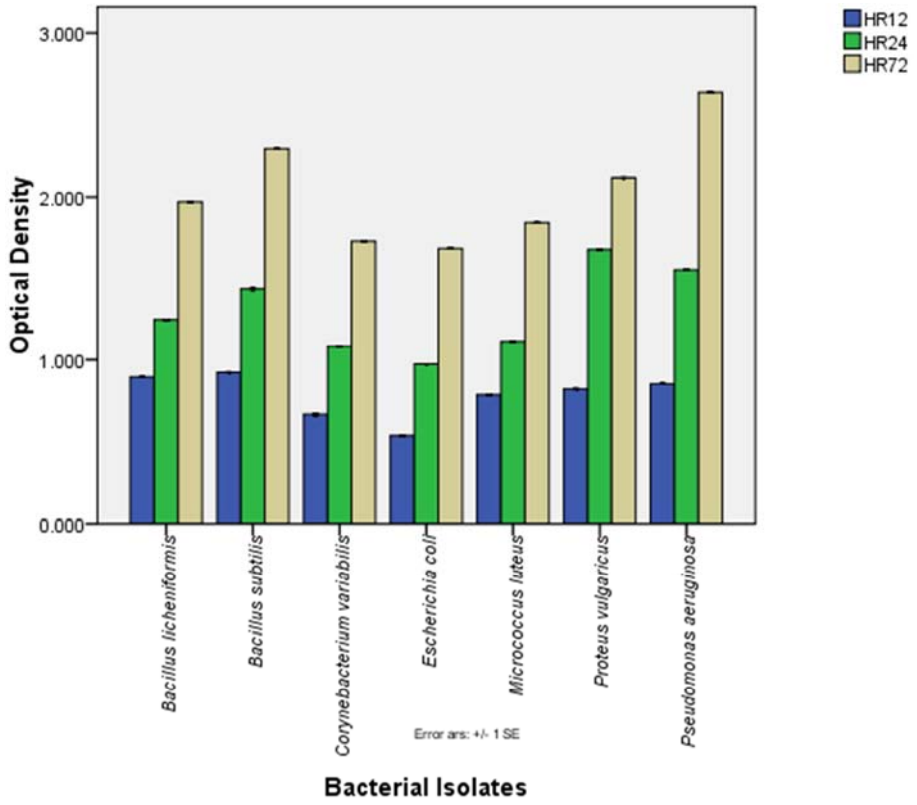


Figure 6. *Optical density of bacterial isolates from contaminated soil at different hours of biodegradation of used engine oil.*

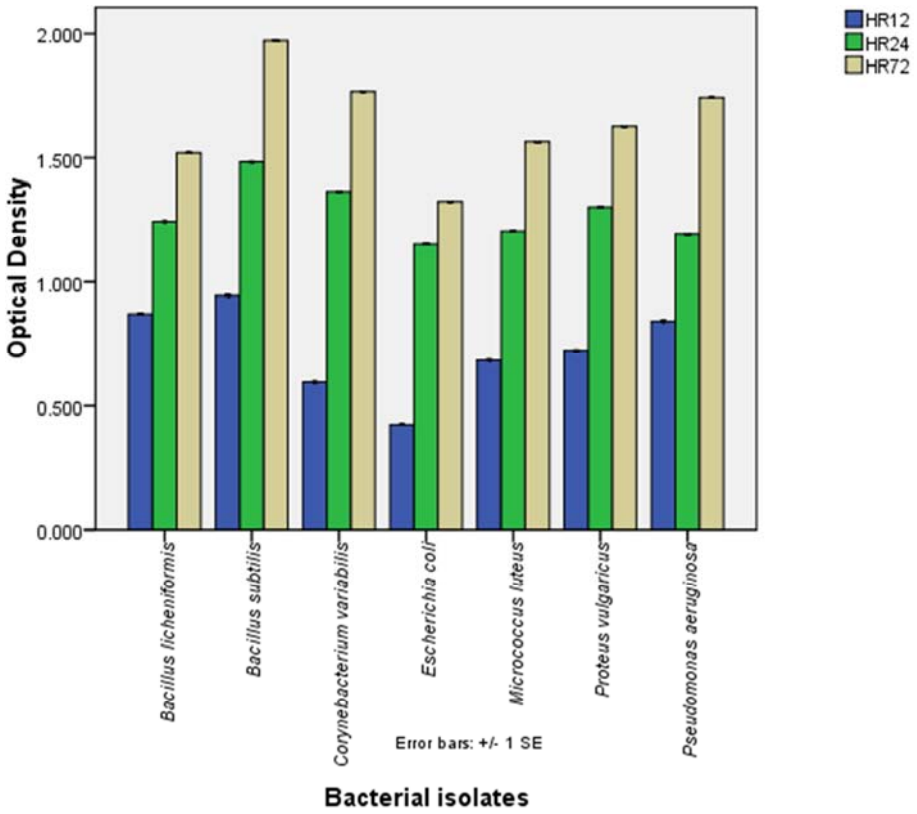


Figure 7. *Optical density of bacterial isolates from non-contaminated (control) soil at different hours of biodegradation of bonny light crude oil.*

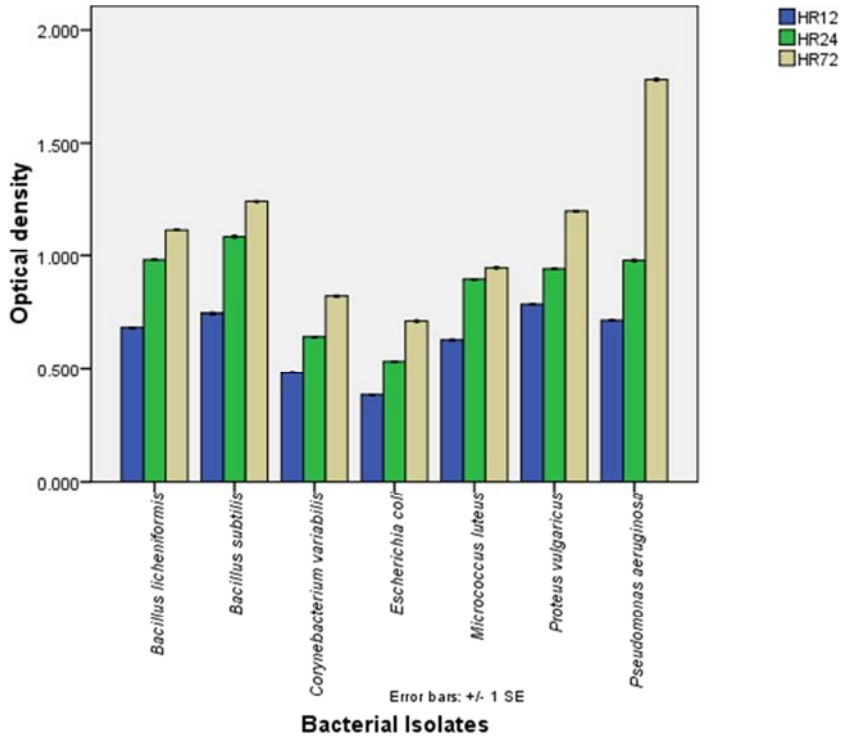


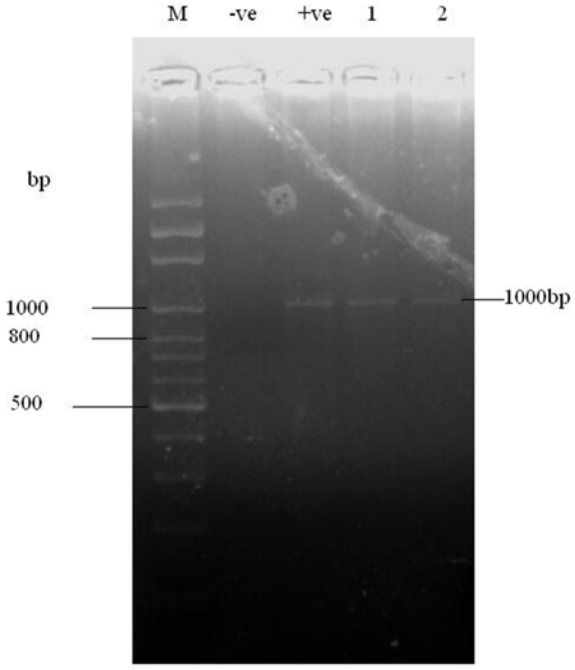
Figure 8. *Optical density of bacterial isolates from non -contaminated (control) soil at different hours of biodegradation of used engine oil.*

3.5. Total Petroleum Hydrocarbon Content of the Crude Oil and Soil Sample

The total petroleum hydrocarbon content of the soils varies with sites. Soil sample A had highest TPH (856,328 Mg/kg), follow by sample B (642,302 Mg/kg) sample C had the least (545,168 mg/kg). The TPH content of control (uncontaminated) sample was 4.90 Mg/kg, while that of bonny light crude oil and used engine oil was 98,346.102 Mg/l and 35,726.80 Mg/l respectively.

3.6. Molecular Identification of Pseudomonas Aeruginosa

Pseudomonas aeruginosa was found to have the same molecular weight with the primer of *Pseudomonas aeruginosa* ATCC 27853. The result is shown in Figure 9.



M = 100bp DNA ladder, -ve = Sterile water,+ve= *Pseudomonas aeruginosa* ATCC 27853

Figure 9. *Gel electrophoresis of PCR products of the confirmed Pseudomonas aeruginosa.*

4. Discussion

The total plate counts of bacteria were higher in uncontaminated soils than contaminated soils. This is likely to be due to the environmental stress and toxicity caused by the hydrocarbons to the bacteria. This finding agreed with the report of [24], that crude oil products contain hydrocarbon that are toxic to microorganisms. The result shows an obvious influence of waste engine oil and crude oil discharge on the microbiological status of soil sample. The significant difference in the total plate count of bacteria in contaminated and control soil samples may be adduced to the fact that only bacteria capable of or synthesize enzymes capable of digesting and/or tolerant to hydrocarbons thrived in the contaminated soils [25]. The growth profile of the isolated microorganisms showed the growth curve exhibiting the lag, exponential, stationary and decline phase which agrees with the report by [26] that the morphology and growth characteristic of the isolated microorganism vary continuously throughout microbial growth phase. The curve showed an exponential phase from 12 to 48 h. All the bacterial isolated from this study were able to produce phytase in varying degrees which agrees with of [27], that soil microbes are able solubilize various forms of precipitated phosphorus. Phytase has been detected in various bacteria, e.g. *Pseudomonas aeruginosa* [28], *Bacillus subtilis* [29], *Bacillus licheniformis* [30], *Escherichia coli* [31] and *Enterobacter sp*. [32].

The maximum phytase activity was more prominent at the 48 h of incubation. It was observed that of all the bacterial isolates, *Pseudomonas aeruginosa* had the maximum phytase activity at the 48 h of incubation while *Proteus vulgaricus* produced the least phytase activity at all the hour of incubation. The pH versus phytase activity profiles of the selected bacteria shows substantial production of phytase at two distinct pH optimum; the highest activity was recorded at pH 5.0 and a second activity peak occur at pH 3.0 which is in accordance with the work of [33]. Maximum activity for phytase production from the selected bacteria was attained at 40°C. According to [34], the phytase molecule has a limited thermal stability and studies have demonstrated that losses in activity begin to occur at around 60°C. *B. subtilis*, *P. aeruginosa*, *P. vulgaricus*, *B. licheniformis*, *M. luteus*, *E. coli* and *C. variabilis* isolated in this study showed evidence of high ability to degrade crude oil as compared to used engine oil and this can be attributed to the presence of saturated alkane with intermediate chain (C10 – C24) length [35] in crude oil in contrast to engine oil.

The biodegradatives potentials shown by the recovered isolates in contaminated soil is believed to have been enhanced by the sufficient availability of phosphorus which is made available by the hydrolyzing effect of phytase on the complex salts of phosphorus that are in insoluble in the soil, and phosphorus is known as one of the limiting nutrient in biodegradation process. The high TPH concentration in contaminated soil could be due to continuous exposure of the soil to used engine oil. This high concentration of TPH makes soil conditions unsatisfactory for microbial growth [36]. The TPH level in the uncontaminated soil (control) was as low as 4.9 mg/kg indicating that hydrocarbons could be present in uncontaminated soils and sediments, as earlier reported by [36]. The *Pseudomonas aeruginosa* isolated from both contaminated and non-contaminated soils were observed to have the same base sequence with the primer of *Pseudomonas aeruginosa* ATCC 27853 used as positive control.

5. Conclusion

This study provides vital information that would lead to selection of biodegradative bacterial species that could be employed for bioremediation of soils polluted with crude and spent engine oils. It is therefore evident that oil-degrading bacteria are abundant in mechanic workshop soils in Akure and they are very efficient tool in Bioremediation.

Abbreviations

MSM, Minimal salts medium; GC, gas chromatographic; TPH, total petroleum hydrocarbon; ATCC, American type culture collection

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